BIOLOGICAL CONVERSION OF BIOMASS TO ALTERNATE FUELS

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1	Thippareddy K.S. and Pushpa Agrawal. Ethanol production from lignocellulosic biomass through anaerobic fermentation.	Journal of Environmental Research and Development, 3 (2) 2008, 395-399.
2	Thippareddy K.S. and Pushpa Agrawal. Two step-fermentation process for the production of ethanol from agricultural waste.	Journal of Environmental Research and Development, 4/4, 2010, 964—969
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4	Thippareddy K.S. and Pushpa Agrawal. "Pre-treatment of agriculture waste using *A. flavus* for the production of ethanol by *S.cerevisiae*"	Journal of Environmental Research and Development, 5 (2), 2010 393-403
5	Thippareddy K.S. and Pushpa Agrawal. "Studies on Characterization of Agriculture waste (rice husk) for the production of ethanol" Journal of Environmental Research and Development Bhopal (India).	Vol.7 (2A) year 2012 Nov-Dec page 1076-1084. ISSN0973-6921
6	Thippareddy K.S. and Pushpa Agrawal. "Characterization Aspects For The Biological Production of Ethanol From Deseeded Sun Flower Heads"	Journal of Environmental Research and Development Bhopal (India). Vol.9 no.2 pp 334-341 year, Nov-Dec 2014.

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FINAL SYNOPOSIS

Needless to say, the world population is increasing at an alarming rate and so is the liquid fuel demand in the transport sector. Global warming, depletion of fossil fuels and increasing price of petroleum-based fuels are gaining great concern and the exigency of the situation has forced the search for alternative, sustainable, renewable, efficient and cost effective energy sources with lesser green house gas emission. Biomass can serve as an excellent alternative source to meet the present and future fuel demands. Any type of fuel generated from biomass is termed biofuel. The two most common and successful biofuels are biodiesel and bio-ethanol which are aimed at replacing mainly the conventional liquid fuels like diesel and petrol.

The aim of the research work was to develop an alternative and cost effective technology for synthesis of renewable and sustainable energy fuels by means of biological conversion of bio-waste. The proposed thesis is broadly classified into seven chapters. They are 1) Introduction, 2) Literature survey, 3) Materials and Methods, 4) Characterization of fuels, 5) Results and Discussion, 6) Statistical analysis 7) Conclusion and scope for future work.

Chapter one "Introduction" deals with study of energy crisis, energy sources, energy short coming, alternative energy sources, biological conversion methods, air pollution, etc. The problem in traditional fuels unable to meet the demand of world is addressed in this chapter. The different energy sources to overcome this situation are clearly explained. The statistics of world energy demand and available energy sources are given with quantification data.

Chapter Two: Literature review: review deals with study of availability of technology to synthesis biofuel by means of biological conversion, such as bacterial conversion, fungal conversionand enzymatic conversion etc.

The hydrolysis methods most commonly used are acid, Alkaline (dilute and concentrated) and enzymatic. The lignin-hemicellulose net work has to be loosened for better amenability of cellulases to residual carbohydrate fraction for sugar recovery. (Chandel et al., 2007). The acid, alkaline or fungal pre-treated lignocellulosics can be saccharified enzymatically to get fermentable sugars. Bacteria and fungi are the good sources of cellulases, hemicelluloses that could be used for the hydrolysis of pre-treated lignocellulosics (Aro et al., 2005). Bioconversion of various biomass sources into ethanol is obtained by different microorganisms. The sugar syrup

obtained after cellulosic hydrolysis is used for ethanol fermentation. In fed batch fermentation the microorganism works at low substrate concentration with an increasing ethanol concentration during the course of fermentation process. Continuous fermentation can be performed in different kind of bioreactors – stirred tank reactors (single or series) or plug flow reactors. Continuous fermentation often gives a higher productivity than batch fermentation, but at low dilution rates which offers the highest productivities. The continuous process eliminates much of the unproductive time associated with cleaning, recharging, adjustment of media and sterilization. Immobilization by adhesion to a surface (electrostatic or covalent), entrapment in polymeric matrices or retention by membranes has been successful for ethanol production from hexoses. (Chandel et al., 2007) Tarique et al., reported as the spent tea being used for the production of biofuels and also cheap source of the energy which can be made easily available to the world. *Aspergillus* grow on spent tea bio-ethanol yield and also done XRD Scanning Electronic microscope (SEM), FTIR characterization (Tariq Mahmood et al, 2010).

Chapter Three: Materials and Methods

The biological agents such as corn cob, rice husk and de-seeded sun flower head were used as raw materials. The chemical composition of the corn cob: Cellulose 42% and hemicellulose 39% lignin 14%.(Chandel et al., 2007). Composition of the rice husk: Cellulose 35%, Hemicellulose 25%and lignin 20%. Rice husk is a source of lignocellulosic biomass that may be utilized as raw material for ethanol manufacturing (Ali Abbas et al., 2010). Sunflower de-seeded head has the presence of combined carbohydrates: lignin 12% galacturonic acid (8%), galactose (3.4%), glucose (1.7%), arabinose (0.63%), xylose (0.7%) and rhamnose (21%) (M. Edrees, et al., 1975).The corn cub, rice husk and de-seeded sun flower head were reduced in size up to 1.2 μm using ball mill then dried at a temperature of 60°C as per suggestion given by [15]**.** The dried substrate was treated with 5% sodium hydroxide solution, washed using distilled water, dried in oven at 60^{o}C and then autoclaved at 15 psi (pounds per square inch) for 20 minutes.

The corn cob, rice husk and de-seeded sunflower head are biomass substrates used in chemical and fungal pretreatments. The samples were sputtered with gold coating in IISC at Bangalore, after gold coating samples were viewed in SEM Hitachi 1500 Japan make. Samples were kept in

two sided carbon tape adhesive to sample holder stub, then the sputtered sample was scanned in Scanning Electronic microscope (SEM).

The corn cob, rice husk and de-seeded sunflower head are biomass substrates used for the physical treatments in the ball milling (make pulverisetteFritesch-6 planetary monomill) working on the principle of impact force. All the experiments were conducted using steel Jar and Steel balls. Steel balls were 100 in numbers, each ball of size 10 mm diameter, each ball weigning 4gm, Steel Jar size 500 ml. Total weight of is the balls 400 gms, Ball-to-powder weight ratio = 15:1, Amount of substrate= 400/15 =26.67 =27 grams. The above corn cob, rice husk and de-seeded sunflower head were milled for 30 minutes at 250 rpm (rotational per minute) at an interval of 10 minutes pause (avoid excess heat), cooled at room temperature. The powder was collected and the size of the particles was determined.

The corn cob, rice husk and de-seeded sunflower heads were subjected to Powder X-ray diffraction (PXRD). Measurements were performed on a PAN-analytical Xpert Pro X-ray diffract meter using CuKr radiation (λ) 0.154 nm) at 40 kV, at a scanning rate of 2° min^{-1}. The difference was observed between treated and untreated substrate. Peaks are sharp in chemical treated and no sharp peaks in untreated substrate were observed.

The corn cob, rice husk and de-seeded sunflower heads powders were used for the infrared (IR) spectra. Samples were collected using a Nicolet IR200 FT-IR spectrometer using KBr pellets, in the range 4000-400 cm^{-1} with 4 cm^{-1}, resolution. To identify a component of certain compounds, they are exposed to high energy such as Infrared Radiation (IR). The reaction results in emission of energy showing the reactions of the molecules, which are automatically plotted to a graph by one of the programs embedded in spectroscopic instruments. Using the generated graph, one can analyze the plot and detect distinctive peaks that can be attributed to the components of the compound. The corn cob, rice husk and de-seeded sunflower heads powders were used for the particle size analysis. (Instrument Microtrac Zetatrac)The powder was weighed to 0.005 grams dissolved in 10 ml distilled water. After dissolving, 3 ml of solution was poured in to the Cuvette of the particle size instrument. Then, as per standard operating procedure, density and refractive index powder include in the display will be shown on the calculated graphs [10, 11].

Substrate was treated with *Aspergillus niger and Aspergillus flavus* (grown in PDA Media) in aseptic condition at pH 4.5 to 7.5 with intervals, of 0.5 and 15^{o}C to 45 ° C with interval of 5°C for 7 days. The conversation to reducing sugar due to the activities of fungi had been estimated by analyzing sugars by DNS method. Thus pre-treated substrate was sterilized and dissolved in 200 ml of distilled water**.** Then aliquots of different (temperature) pH grades in the range of 4.5 to 7.5 were developed. To each grade, the microorganisms like *Saccharomyces cerevisiae 181, Saccharomyces cerevisiae 180, Zymomonas moblis* were cultured in respective media 100ml quantities for 48 hours, incubation at 30^{o}C are inoculated [7, 8and 9]. Ethanol concentration was measured using UV- visible-double beam spectrophotometer (Double Beam, Elico company, model no Elico -SL 164). Ethanol was analyzed by the dichromate oxidation method which is based on the complete oxidation of ethanol by dichromate in acidic medium. Reducing sugars were determined by photo metrically using dintrosalicylic acid reagent (Systronics Colorimeter: model No 115).

Chapter Four: Results and Discussion

The corn cob substrate was subjected to *Aspergillus niger* for bioconversion process. Initially, the substrate was converted to reducing sugars and the resultant substrate was subjected to produce the ethanol using other microorganisms. Scanning Electron Microscope Images show various organisms at different pHs converting the substrate to reducing sugars and in turn to ethanol.

1. Fourier Transform IR (Ftir**) :** A graph shows two distinctive peaksand after analyzing the plot, one peak corresponds to Hydrogen (H) and the other to Oxygen (O_2); thus, it can be safely said, that it is H_20 or water molecule. Molecules that react with IR always exhibit the same distinguishing peak of energy, so they can easily be identified from the graph. The corn cob of the spectrum and the assignment of the peak 600,790,750cm^{-1} due to C—H bending vibration A peak 1000, 1250, shows C—O (str) Vibration respectively, 1500, 1680, due to N—H stretching vibration, a peak of 1800, 2050Cm^{-1} C=O (str) of Carobonyl group. A peak due to C—H stretching vibration appears to 2470, 2990 and a broad peak 3495, shows O— H Stretching due to alcoholic group.

2. X-Ray Diffraction: The lattice plane at 2θ equal to 24^0 represents the amorphous cellulose, while the lattice plane at 2θ equal to 22^o represents the crystalline cellulose. Qualitative evaluation of the difractograms clearly shows that the crystalline plane of the pre- treated sample is sharper and of higher intensity than that of untreated corn cob. The higher is crystallinity of the pre-treated sample. The crystallinity index was 0.67 and 0.7 for the pre-treated sample and untreated corn cob, respectively. The crystalinity index of the pre-treated sample was higher because of the hemicellulose and lignin, which are amorphous materials.

3. Particle Size Analysis: Microtrac has enhanced the Nanotrac instrument line to include Zeta Potential measurement and Particle Size. The corn cob substrate of the particle size 1.233 microns in the given sample [12, 13].

Reducing sugar and ethanol production by showing different pHs and temperatures is shown below in the graphs.

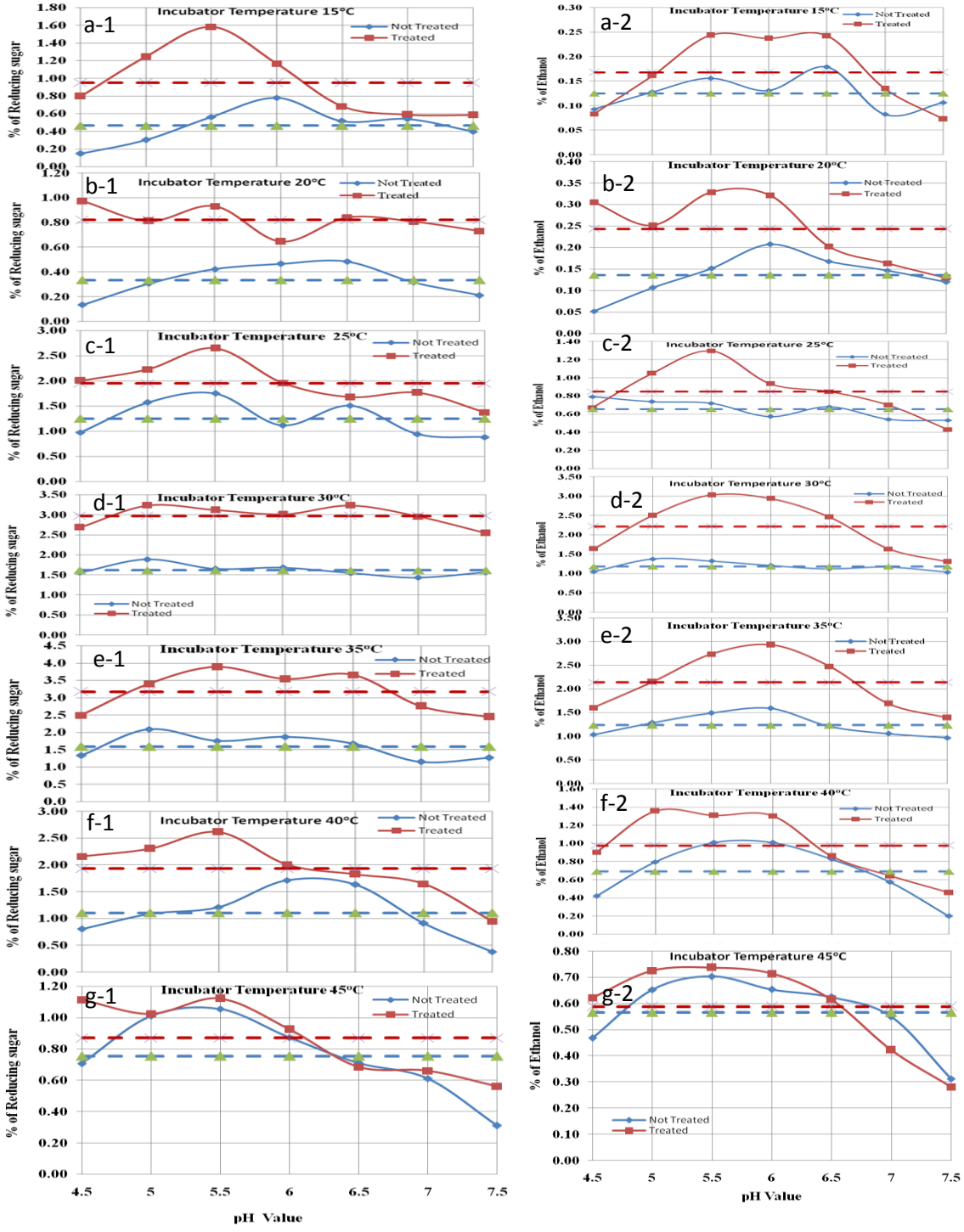

Figure: 1. Effect of temperature and pH Values on (1) reducing sugar(*Aspergillus flavus*) and (2) % of Ethanol (*Zymomonas mobilis, Saccharomyces cerevisiae 180 and Saccharomyces cerevisiae 181*)produced from corn cob at the temperature of a.15^{o}C, b.20 ^{o}C, c.25 ^{o}C, d.30 ^{o}C, e.35 ^{o}C, f.40 ^{o}C, g.45 ^{o}C (Blue line untreated, Red line treated, blue dotted line – Average untreated and red dotted line-Average treated line)

The Figure. 1. shows the effect of pH on reducing sugar (a-1) and % of ethanol (a-2) from treated and untreated corncobs using *Aspergillus flavus* (reducing sugar) and *Zymomonas mobilis*, *Saccharomyces cerevisiae* 181and *Saccharomyces cerevisiae 180* (% of ethanol) at seven days exposure in solution media. Both treated and untreated curves behavior could be categorized into three sections. First-section is increasing reducing sugar as well as ethanol with increasing pH values of the solution. They reach peak at pH 5.5 then stabilize in second section upto pH 6.5 and in last section the yield of both decrease with increasing pH values. In all cases the treated specimens show higher yield compared to untreated. In stabilized pH values (pH. 4.5-6.5) the enzyme activity is more on carbon source with xylanase activity. The xylanseses in acid group could be a key enzyme to improve the bioconversion of lignocellulosic materials. The pretreatment would reduce the lignin content and crystalinity of cellulose and increase surface area. The alkaline treatment was identified as suitable to result in quantitatively yields of reducing sugars. Enzyme activities are found to be higher with the mycelia inoculums compared to the spore inoculums. The enzyme production increases growth of the fungus which depletes the available nitrogen and the system becomes lignolytic, for the necessary activation. The effect of high and low temperature on synthesis of cellulose protein and maximum cellulase activity at 30^{o}C and 35^{o}C is shows figures 1. (a-2) production of ethanol by *Saccharomyces cerevisiae* 181, *Saccharomyces cerevisiae 180 and Zymomonas mobilis* at different temperatures and different pHs. At pH 5.5 and 6.5 peak height increased for treated and untreated response at temperatures 30^{o}C and 35^{o}C. (Blue line untreated, Red line treated, blue dotted line – Average untreated and red dotted line-Average treated line) The Saccharifcation over the period converts the glucose available by using *Saccharomyces cerevisiae 181*, *Saccharomyces cerevisiae 180 and Zymomonas mobilis to ethanol*. *Zymomonas mobilis and Saccharomyces cerevisiae* were found to be successful in fermenting the hydrolyzed waste starch. Previous reports on the nutritional requirements for the optimal growth factors and minimizing the inhibitory effects of ethanol *Zymomonas mobilis* organism should grow in high sugar concentrations and high ethanol tolerance. It should also produce ethanol at as fast a rate as possible and if vacuum distillation is needed to distill off the ethanol [14, 15, 16 and 17].

CHAPTER FIVE- STATISTICAL ANALYSIS

The software used is Minitab 16 version. The Taguchi method, ANOVA, Regression analysis and contour plot graph emphasizes a mean performance characteristic value close to the target value rather than a value within certain specification limits, thus improving the product quality. Additionally, Taguchi's method for experimental design is straightforward and easy to apply to many engineering situations, making it a powerful yet simple tool. It can be used to quickly narrow down the scope of a research project or to identify problems in a manufacturing process from data already in existence. Also, the Taguchi method allows for the analysis of many different parameters without a prohibitively high amount of experimentation.

51. The effect of *Aspergillus flavus* producing reducing sugar with and without chemical treatment of corncob

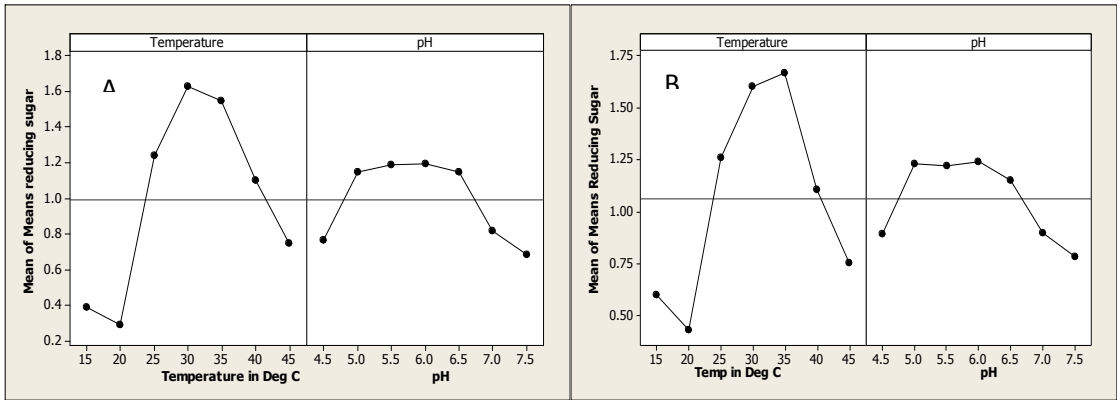

Figure 2: Means of means reducing sugar production A) untreated B) Treated

Temperature has complex effect on enzyme activity and hence Saccharifcation. It affects the speed of molecules, the activation energy of the catalytic reaction and thermal stability of the enzyme. Generally, saccharification increases with temperature upto the optimum after which it declines. The pH of a solution has several effects on the structure and activity of enzyme is amphotoeric molecules containing a large number of acid and basic groups, mainly situated on their surface. The charges on these groups vary, according to their acid dissociation constants, with the pH of the solution as shown in Figure (2. A and B) [18, 19].

Effect of temperature on Saccharifcation is shown in Taguchi model, Figure (2.A and B). Figure (2.A) untreated an optimum value of 1.038 % of reducing sugar from *Aspergillus flavus* was produced at normal temperature (35^{o}C) and higher pH (6) value and 2.B) Treated an optimum

value of 1.34 % of reducing sugar from *Aspergillus flavus* was produced at normal temperature (35^{o}C) and higher pH (6) value.

Table 1: Regression analysis

Reducing Sugar for untreated = 0.903745 + 0.00903556 Temperature - 0.0304432 pH
Reducing Sugar for treated = 1.37232 + 0.0119349 Temperature -0.0765702 pH

The regression analysis, the regression model shown in equation treated and untreated (table 1), the (table 2.)R-sq (82.70%) indicates that parameters such as temp and PH are highly co related with reducing sugar for untreated response. The regression model shown in equation (table 1) the and (table 3.) R- sq (93.72%) indicates that parameters such as temp and PH are highly co related with reducing sugar for treated response [20].

Table 1: Analysis of Variance for *Aspergillus flavus* (R sUT), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P	%
Temperature	6	9.84791	9.84791	1.64132	24.66	0.000	71.1
pH	6	1.60282	1.60282	0.26714	4.01	0.004	11.6
Error	36	2.39615	2.39615	0.06656			17.3
Total	48	13.84688					100
S = 0.257991 R-Sq = 82.70% R-Sq(adj) = 76.93%							

Table 2: Analysis of Variance for *Aspergillus flavus* (RS T), using Adjusted SS for tests

Source	DF	Seq SS	Adj SS	ADj MS	F	P	%
Temperature	6	43.8160	43.8160	7.3027	80.72	0.000	84.6
pH	6	4.7564	4.7564	0.7927	8.76	0.000	9.2
Error	36	3.2570	3.2570	0.0905			6.2
Total	48	51.8294					100.0
S = 0.300785 R-Sq = 93.72% R-Sq(adj) = 91.62%							

In the table (Table 1) P and F distributions of ANOVA indicate that both the temperature and pH are significant influencing factors for reducing sugar for treated response. The interactions between the above factors have significant variation on the production of reducing sugar. The above observation in the table temperature (P 71.1%) and (pH 11.6%) was more reinforcement has great influence on the production of reducing sugar. The reinforcement is influencing comparatively less (pH 11.6%).which indicates that there is no appreciable increase in

production of reducing sugar by increasing the reinforcement (pH). The reliability of the experimental result was found upon the contribution of error which 17.2% was below 50% maximum error limit, thus implying the insignificance of experimental error. The significance of each factor was ranked according to its percent contribution temperature and pH. (Table 2)The P and F distributions of ANOVA indicate that both the temperature and pH are significant influencing factors for reducing sugar for treated response. The interactions between the above factors have significant variation on the production of reducing sugar. The above observation in the table temperature (P 84.6%) and (pH 9.2%) was that great reinforcement has great influence on the production of reducing sugar. The reinforcement is influencing comparatively less (pH 9.2%).which indicates that there is no appreciable increase in production of reducing sugar by increasing the reinforcement (pH). The reliability of the experimental result was found upon the contribution of error, 6.2% was below 50% maximum error limit, thus implying the insignificance of experimental error. The significance of each factor was ranked according to its percent contribution temperature and pH. [19]

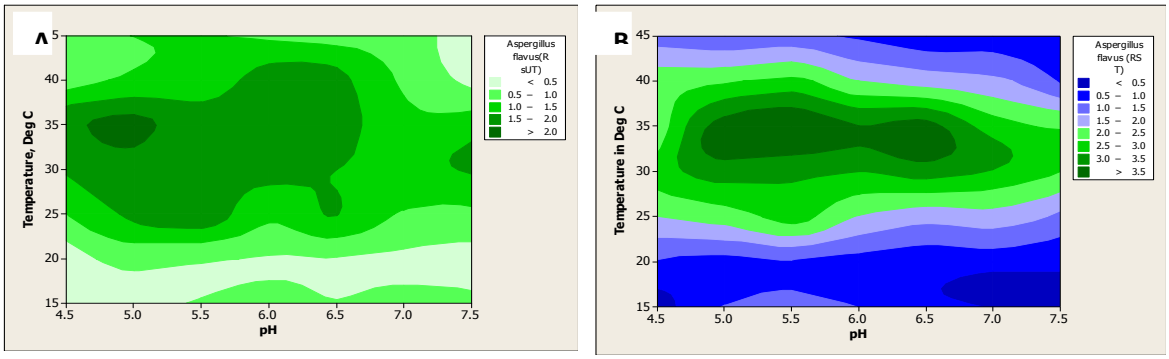

Figure: 3. A) Untreated and B) Treated, from contour plot of temperature vs pH, reducing sugar production

Figure 3 A the contour green colour to light green colour (2.0 to 0.5 amount of reducing sugar decreased where temperature increases the production of reducing sugar reduced). The contour plot of temperature vs pH, it was evident that at normal temperature of 35^{o}C and at lower pH value (6 pH), the percentage of *Aspergillus flavus* producing reduced sugar has increased because the growth of corn cob on Aspergillus flavus degrades the cellulose, hemi cellulose and the lignin into reducing sugar. Figure 3. B the contour green colour to blue colour(3.5 to 0.5 amount of reducing sugar decreased, when temperature increases, the production of reducing sugar reduced). From the contour plot of temperature vs pH, it was evident that at normal

temperature of 35^{o}C and at lower pH value (5 pH), the percentage of *Aspergillus flavus* producing reduced sugar has increased because the growth of corn cob on *Aspergillus* flavus degrades the cellulose, hemi cellulose and the lignin into reducing sugar. Design of Experiment is one of the important and powerful statistical techniques to study the effect of multiple variables simultaneously and involves a series of steps which must follow a certain sequence for the experiment to yield an improved understanding of process performance [21, 22].

All designed experiments require a certain number of combinations of factors and levels be tested in order to observe the results of those test conditions. Taguchi approach relies on the assignment of factors in specific to determine those test combinations.

The DOE process is made up of three main phases: the planning phase, the conducting phaseand the analysis phase. A major step in the DOE process is the determination of the combination of factors and levels which will provide the desired information.

Analysis of the experimental results uses a signal-to-noise ratio to aid in the determination of the best process designs. This technique has been successfully used by researchers in the study production of reducing sugar and *Aspergillus flavus* and *Aspergillus niger* conversion capacity in untreated and chemical treated substrate to reducing sugarand production of ethanol by using microorganisms are ethanol production using *Zymomonas mobilis*, *Saccharomyces cerevisiae180* and *Saccharomyces cerevisiae* 181 highly influenced temperature and pH for the coefficient of these factors. These methods focus on improving the design of experiment processes. In the present work, a plan order for performing the experiments was generated by Taguchi method analysis of parameters was done using ANOVA technique. This method yields the rank of various parameters with the level of significance of influence of a factor or the interaction of factors on a particular output response [23].

Chapter Six: Conclusion and scope of future work**.**

From the results, it is evident, that fermentation technique can yield relatively higher quantity of ethanol compared to any other combination of treatments used in the present experiment. Further, Z*ymomonas mobilis* has more affinity to convert the reducing sugars to ethanol by fermentation. It was also observed, that the chemically-treated substrate (corn cob), with sodium hydroxide, is more amenable for *Aspergillus niger* activities and yields more reducing sugar

compared to untreated substrate and thus, enhances the available reducing sugar for Z*ymomonas mobilis* fermentation activity to produce ethanol. *Saccharomyces cerevisiae* has more affinity to convert the reducing sugar to ethanol by fermentation. The degrading of the lignocelluloses material by enzymatic activity *Aspergillus flavus* are the fungus by which the reducing sugar were obtained in the form of glucose and then subjected to fermentation process to produce the ethanol. Thus *Aspergillus flavus* enhances the available reducing sugar by the degradation of cellulose and lignocelluloses for *Saccharomyces cerevisiae* fermentation activity to produce ethanol.

Scope of future work: The important issue that has to be addressed affirmatively is that the bio-ethanol production needs an economical approach to address the global fuel needs. Research efforts are needed to design and improve the process, which would produce sustainable and economically feasible transportation fuel.

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